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The aim of this study was to isolate, identify and determine the antimicrobial susceptibility of *Clostridium perfringens* (CP) isolates from acute necrotic enteritis of broiler chickens. Broiler carcasses diagnosed as necrotic enteritis (NE) were sampled, subjected to microbial tests and 40 isolates were identified according to standard procedures. The antimicrobial susceptibility of CP isolates to 20 antibacterial agents was then determined. The results show widespread resistance among CP isolates. The most frequent resistance was observed to neomycin sulfate (87.5%), and then to lincomycin and tetracycline (both 80%). No isolate was resistant to chloramphenicol and the least frequency of resistance was observed to vancomycin (10%), sulfamethoxazole+trimethoprim (17.5%), and penicillin (20%). All isolates were multiple drug resistant types. There were 39 resistant patterns among the CP isolates, 95% of which were distributed in 38 resistant patterns. These multiple and variable resistance patterns observed among the CP isolates, even among different isolates from one farm, demonstrate a challenge for veterinarians in the field to choose the correct compound to combat the occurrence of NE.

challenge for countries in which the ban is in place to find an effective antibacterial agent to combat this

Clinical necrotic enteritis (NE) is one of the deadly diseases. A number of studies have shown the role of bacterial diseases, found primarily in young chickens, of antibiotic-supplemented feeds on the development of Clostridium perfringens (CP) type A and, resistant strains to antibacterial agents (Redal, 1978; to a lesser extent, type C (Prescott et al., 1978; Shane 1978; Summanee et al., 1993). This resistance may develop because the use of antibiotics in feeds has led to the selection of resistant bacteria (Redal, 1978). Both CP types are known to produce toxins: type A, alpha toxin and type C, both alpha and beta toxins (Shane et al., 1985; Van Immerseel et al., 2004). In spite of having knowledge about many predisposing factors to NE (Williams, 2005), when facing the disease, veterinarians have to administer an appropriate antibiotic to birds to reduce the mortality rate, as well as other effective in the prevention and treatment of the disease. After the ban on the use of growth promoter antibiotics, detrimental effects of the disease. Therefore, determining and ionophore anticoccidials in the European Union, the antimicrobial susceptibility of CP isolates from NE (EU), NE has become one of the most important threats to the broiler industry in the EU (Casewell et al., 2003; recovered from acute clinical NE cases were characterized Gravee et al., 2004; Chalmer et al., 2007). In the US, for their antimicrobial susceptibility patterns.

promoter antibiotics, different Clostridial diseases

began to increase (Shane, 2004). However, in some countries, where growth promoter antibiotics and ionophores are still utilized for poultry, the occurrence of NE is not as common as in EU countries, which have banned the use of these drugs. It still remains

isolation and identification of Clostridium perfringens (CP) of all broiler chickens diagnosed as necrotic enteritis (NE) (the presence of typical

fibrinonecrotic lesions in the mucosal membrane of the intestines) were sampled and subjected to microbiological tests. The intestinal serosal surface was sterilized with a hot spatula. An incision was then made and a part of the mucosal surface of the intestine was taken by a sterile loop for a smear and gram stain. Identification of the bacteria was performed according to procedures described by Summanan et al. (1993), Quinn (1994), Miller (1998). A presumptive diagnosis of CP was made for Gram-positive, spore-containing bacteria. These samples then were streaked onto blood agar plates (BA) and placed in anaerobic jars (Merck, Germany) containing commercial gas pack (Anaerocult A, Merck). The jars were closed and incubated at 37°C for 48 h. The indicator strips (Anaero-test, Merck) were included in each jar to confirm the anaerobic conditions. After 48 h, the BA plates were examined for colony morphology. Observation of large, smooth and round colonies with 2-4 mm in diameter having double hemolysis (complete hemolysis in the inner zone and incomplete hemolysis in the outer zone) were considered as presumptive diagnosis of CP. The colonies were checked by Gram-staining of the colonies observed under the microscope. The suspected positive samples were screened for lecithinase, lipase, urease, and indole production, motility, and reverse-CAMP test. Finally, the suspected colonies were cultured on Tryptone Sulfite Neomycin (TSN; Merck) agar plates. TSN-inoculated plates were incubated anaerobically at 37°C for 18 h. Dark-centered colonies were considered as containing CP.

Antimicrobial susceptibility test

The susceptibility of 40 CP isolates to a panel of antimicrobial agents was determined as previously described (Quinn et al., 1994). The antimicrobial agents that were tested, and their concentrations (µg) were as follows: difloxacin (10), ofloxacin (5), norfloxacin (10), enrofloxacin (5), nalidixic acid (30), flumequine (30), penicillin (10), ampicillin (10), amoxi-clav (30), neomycin (30), gentamicin (10), lincomycin (30), lincospectin (15/200), erythromycin (10), tylosin (30), chloramphenicol (30), tetracycline (30), colistin (10), vancomycin (30) and trimethoprim-sulfamethoxazole (1.25/23.75). In this study, the CP isolates with intermediate susceptibility classification were considered not to be resistant to that drug and the multi-resistance was defined as resistance to more than one drug.

Results

In the present study, the resistance to antimicrobial compounds was found to be widespread among the CP isolates. The most frequent resistance was observed to neomycin sulfate (87.5%), and then to lincomycin and

tetracycline (both 80%; Table 1). No isolate was resistant to chloramphenicol and the least frequency of resistance was observed to vancomycin (10%), sulfamethoxazole+trimethoprim (17.5%) and penicillin (20%; Table 1). All isolates were resistant to more than one antibacterial agent. More than 50% of isolates were resistant to more than five drugs and one isolate (2.5%) showed multiple resistances to more than 14 drugs. There were 39 resistant patterns observed to 20 tested antibacterials among the CP isolates that were tested. Thirty-eight (95%) isolates each showed an individual resistance patterns. Only two isolates (5%) showed an identical pattern of resistance.

Discussion

Different antibacterials have been used for the treatment, or as in-feed growth promoters for the prevention, of NE outbreak in poultry (Prescott et al., 1978; Hamdy et al., 1983). The susceptibility of CP isolates to different sources of antibacterials has been studied by many and variable results have been observed. Junget al. (1983) evaluated the sensitivity of 50 CP isolates from human feces to cephotaxim, fosfomycin, penicillin-G and vancomycin. They observed no resistance to pen-G or cephotaxim, but did observe variable resistance to other agents. Devriese et al. (1993) studied the minimum inhibitory concentration of seven growth promoter antibacterials against 95 CP isolates from poultry, pigs and calves. These researchers found resistance to bambamycin and flavomycin (flavophospholypol) and susceptibility to flavoparcin, avilamycin, and salinomycin among all 95 isolates. Resistance to tylosin and virginiamycin

Table 1: Antimicrobial susceptibility test results of 40 *Clostridium perfringens* isolates from cases of necrotic enteritis.^a

	Antimicrobial drugs	S	I	R
1	Vancomycin (Vc)	90	0	10
2	Erythromycin (Er)	2.5	67.5	30
3	Tylosin (Ty)	25	47.5	27.5
4	Amoxi-Clav (Amx)	70	0	30
5	Ampicillin (Amp)	40	32.5	27.5
6	Penicillin (Pen)	80	0	20
7	Gentamicin (Gen)	47.5	0	52.5
8	Flumequine (Flu)	52.5	7.5	40
9	Colistin (Col)	12.5	47.5	40
10	Tetracycline (Tet)	7.5	12.5	80
11	Chloramphenicol (Chl)	82.5	17.5	0
12	Lincomycin (Lin)	20	0	80
13	Linco-spectin (LP)	57.5	10	32.5
14	Ofloxacin (Ofx)	50	10	40
15	Norfloxacin (Nor)	67.5	10	22.5
16	Enrofloxacin (Nfx)	37.5	30	32.5
17	Neomycin (Neo)	5	7.5	87.5
18	Nalidixic acid (NA)	35	12.5	52.5
19	Difloxacin (Dfx)	70	2.5	27.5
20	Trimethoprim- Sulfamethoxazole (SXT)	82.5	0	17.5

S = Susceptible, I = Intermediate Susceptible, R = Resistant

among isolates from different sources, and resistance to the anaerobic conditions that are required for to bacitracin in some of poultry and calf isolates, was observed. Since culture and antimicrobial also observed. Cummings et al. (1995) conducted susceptibility tests for anaerobic CP are not routinely farm survey and found resistance to lincomycin and used in diagnostic laboratories of this country, in case bacitracin and sensitivity to penicillin. Sasaki et al. (2001) isolated some *Clostridium* species from diseased cattle and reported a 71% resistance to antibiotics and, therefore, the rise of resistance to CP. tetracycline in the CP isolates. Martel et al. (2004) studied the sensitivity of CP isolates, which had been isolated from 31 different Belgian broiler farms, to 12 of CP isolates circulating in broiler farms and the antibacterials and reported a high level of resistance to lincomycin and tetracycline. Johansson et al. (2004) observed 76%, 29%, and 10% resistance to tetracycline. Multiple drug resistant (MDR) types are among CP isolates from Sweden, Norway, and Denmark, respectively. The high level of resistance to tetracycline in Sweden is interesting because this antibiotic was rarely used in Swedish broiler farms. Devriese (1981) found different drug resistant patterns against macrolide-lincosamide and streptogramin in CP isolates of animal origin. Tansupha et al. (2005) Kathar et al. (2006) studied the prevalence of tetracycline resistant genes in 124 CP isolates from *Clostridium perfringens* isolated from the feces of humans and pigs, dogs in the United States and found a relatively high prevalence of *in vitro* resistance to tetracycline. That among 62.7% of antimicrobial resistant strains, high level of resistance to lincomycin and tetracycline 39.3% were resistant to a single drug and 23.4% were also observed among the CP isolates in this study. MDR strains; of 47 MDR strains, 63.8% were derived The high level of resistance to lincomycin can be attributed to resistant genes that had not been detected and Transfer of tetracycline resistance has already been documented in *Clostridia* (Tally and Malamy, 1982). erythromycin, josamycin, tetracycline and, in one case,

In a survey performed from 1986 to 2002 in against chloramphenicol. Roodal et al. (1978) also northern Europe, 100% of CP isolates were found to be observed CP isolates that were MDR strains. These sensitive to vancomycin (Johansson et al., 2004). In isolates were resistant to tetracycline, erythromycin, this study, a 90% sensitivity was observed in the CP clindamycin and lincomycin. However, none of the isolates to vancomycin. Tansuphasanti et al. (2005) isolates were resistant to penicillin or chloramphenicol. examined the antimicrobial susceptibility among 201 These resistant patterns are very similar to the results CP isolates from the feces of humans and pigs, food obtained in this research. Roodal et al. (1978) also found and other environmental sources. These research that resistance to erythromycin was always associated showed resistance to tetracycline (56.2%) followed by with resistance to lincomycin and clindamycin. In this imipenem (24.9%), metronidazole (9.5%), penicillin G study, all the isolates were MDR strains, nine (22.5%) (9%), vancomycin (4.5%), chloramphenicol (3%) and isolates were resistant to more than ten antibacterials, ceftriaxone (1%) among the isolates. Most of the and one (2.5%) isolate showed resistance 140 isolates from pig feces (78%), the environment antimicrobial agents. It should be noted that resistance (72.7%), human feces (44.9%) and food (28%) showed patterns are local phenomenon and using antibacterials resistance to tetracycline. The low level of resistance according to patterns of other regions may be vancomycin and penicillin G observed in this study misleading and inappropriate.

was comparable to findings of Tansuphasiri et al. (2005). In a study by Johansson et al. (2004), 100% of anaerobic bacteria to antibacterials have been studied by some researchers (Finegold, 1989; Root et al., 1978). Root et al. (1978) CP isolates, while a much lower susceptibility was observed to ampicillin. It has been shown that plasmids are the cause for resistance of bacteria to many kinds of antibiotics. Since plasmids

The reason for sensitivity to some antibiotics can be transferred between bacteria of the same, be explained by the level of their usage in poultry farms other, species, and they can carry with them the (Tansuphasiri et al., 2005). In this study, a high level of resistance genes to many antibacterials, resistance may sensitivity to vancomycin and penicillin G was become widespread. Finegold (1989) specified other observed. These antibiotics are not used in Iranian types of resistance encountered in anaerobic bacteria poultry farms. Likewise, tetracycline, which is a including the following: the production of beta- commonly used antibiotic in Iranian poultry farms, was lactamase enzymes, inactivating enzymes such as the drug to which a very high resistance was observed chloramphenicol acetyltransferase, plasmid-mediated One major drawback in monitoring of resistance to C. Per transferable MDR, changes in porin molecules in the

outer membrane of the bacterial cell, decreased uptake of drug by other mechanisms, changes of the target organs such as penicillin binding proteins and a reduction of the antibiotic to an active intermediate product.

The multiple and variable resistance patterns observed in this study among the CP isolates, even among different isolates from the same farm, demonstrate the challenge faced by veterinarians in the field in choosing the correct compound to combat NE. The use of automatic or semi-automatic systems to identify the CP isolates, performing antimicrobial susceptibility test and evaluating an appropriate number of field samples could all play a part in determining a more accurate resistance pattern of an affected flock.

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